

HPV DNA is Associated with a Subset of Schneiderian Papillomas but Does not Correlate with p16^{INK4a} Immunoreactivity

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Abstract This study investigated the role of human papillomavirus (HPV) in Schneiderian papillomas (SPs) to determine whether HPV is associated with the pathogenesis of particular histologic subtypes and whether p16^{INK4a} can be used as a surrogate marker for HPV detection. Twenty-seven papilloma specimens (19 inverted [IPs], 6 exophytic [EPs], 1 oncocyctic [OP] and 1 mixed) were collected from 23 patients. Purified SP DNA extracts were tested for HPV by PCR using GP5 +/GP6 + primers; HPV genotyping was performed by dot blot hybridization. PCR positive specimens were screened for HPV by biotinyl-tyramide-based chromogenic in situ hybridization (CISH). Immunohistochemistry (IHC) for the HPV L1 capsid protein and for p16^{INK4a} was performed on all specimens. HPV was detected by PCR in 16/27 (59.3%) SPs; 9/19 (47.4%) IPs; 6/6 (100%) EPs [$p = 0.051$], and 1/1 (100%) mixed SP. HPV was not detected in the single OP. High risk genotypes were detected in 4/9 IPs (44.4%) and 0/6 EPs (0%) [$p = 0.10$]. Seven of 16 PCR positive SPs were also CISH positive for HPV: 5/6 EPs (83.3%) and 1/9 IP (11.1%) [$p = 0.01$]. IHC for the L1 capsid protein was positive in 2 SPs (1 EP and 1 mixed). p16^{INK4a} staining was seen in 14/16 (87.5%) PCR positive SPs and in 10/11 (90.9%) PCR negative SPs ($p = 1.00$). In summary, this study demonstrates a strong association between HPV and EPs, however, its role in IPs remains less well-defined.

Further, p16^{INK4a} is not a useful surrogate marker for HPV detection across the various SPs.

Keywords Schneiderian papilloma · Sinonasal papilloma · Human papillomavirus · p16^{INK4a}

Introduction

Sinonasal or Schneiderian papillomas (SPs) are benign neoplasms that originate from the Schneiderian mucosa; an ectodermally derived ciliated respiratory mucosa of the nasal cavity and paranasal sinuses [1]. Although known by a variety of names SPs, have been classically divided into three histologic subtypes: (1) fungiform or exophytic (EP) (2) inverted (IP) and (3) cylindrical cell or oncocyctic (OP) papillomas [2, 3]. Etiological investigations of SPs began in the 1980s, with a viral etiology, specifically human papillomavirus (HPV), being of great interest [4]. To date HPV has been detected in EPs and IPs with HPV6 and 11 being the most consistently detected genotypes [1, 4–6]. The rates of HPV detection are highly variable with EPs demonstrating a higher HPV detection rate compared to IPs [6–10]. HPV has not been detected in OPs [6–9].

Clinically, SPs can be difficult to manage as they have a tendency to recur if not completely excised [3, 11]. Beck et al. demonstrated that both the extent of surgical excision and presence of HPV were important in predicting recurrence [12]. Importantly, SPs have also been associated with malignancy [3, 11, 13–15], with IPs and OPs being best documented [13–15]. However, there have been only case reports of EPs and carcinomas [16, 17]. It is this association with malignancy and a high rate of recurrence that drives the interest in understanding the etiology of SPs. If a causative role for HPV in SPs, particularly the IP subtype, is established

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this would have significant implications in surveillance and treatment; in many ways perhaps paralleling the advances seen in cervical cancer screening and prevention.

The p16^{INK4a} protein, a cyclin dependent kinase inhibitor, is upregulated in cells infected with high-risk HPV genotypes [18]. Hence, p16^{INK4a} has been shown to be a surrogate marker for HPV detection in head and neck cancers particularly tonsillar carcinomas [19]. It is also used as a biomarker in aiding the diagnosis of cervical neoplasia [20]. There have been many studies geared towards detecting HPV in SPs by using both PCR and in situ hybridization, however, the literature on p16^{INK4a} and SPs is relatively sparse [21, 22]. The objectives of this study were three-fold: What proportion of SPs in a Vermont population are HPV positive? Does HPV correspond with particular SP histologic subtypes? Can p16^{INK4a} be used as a surrogate marker for HPV positive SPs?

Materials and Methods

Case Selection

With Institutional Review Board approval, 27 formalin-fixed, paraffin-embedded (FFPE) SP specimens (1991–2008) from 23 patients were collected from Fletcher Allen Health Care archives. The 23 patients consisted of 7 females and 16 males with ages ranging from 16 to 94 years (mean = 58.9; median = 60; SD = 16.6). SPs were categorized into their standard histologic subtypes resulting in 19 IPs, 6 EPs and 1 OP (Fig. 1). In addition 1 SP was described as mixed as it displayed features of both an IP and an EP. Mild dysplasia was noted in two EPs. Multifocal moderate dysplasia and focal severe dysplasia were seen in 2 IPs.

DNA Extraction

Up to ten 5 µm sections were cut from FFPE tissue blocks into micro-centrifuge tubes. Tissues were dewaxed with xylene followed by ethanol rinses. DNA was extracted using a combined proteinase K digestion and mini-column purification procedure (DNeasy tissue kit, Qiagen, Valencia, CA). DNA concentration was estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Detection of HPV by PCR

Purified SP DNA extracts were tested for HPV using GP5+/GP6+ PCR primers. Two PCR strategies were applied, a ‘touchdown’ and ‘slow ramping’, as previously described [23]. This widely used assay detects at least thirty-seven different alpha-genus HPV types, which are the types most commonly associated with anogenital lesions.

Identification of Genotype by Dot Blot Hybridization

HPV positive specimens were identified by the detection of a 150 base-pair amplicon after electrophoresis in a 2.0% ethidium bromide stained agarose gel. HPV genotype was identified by dot blot hybridization of PCR products with genotype-specific oligonucleotide probes [24].

Detection of HPV by Chromogenic In Situ Hybridization

PCR positive specimens were screened for HPV by biotinyl-tyramide-based chromogenic in situ hybridization (CISH) as previously described [24, 25]. Briefly, 5 µm section FFPE specimens were dewaxed through xylene and

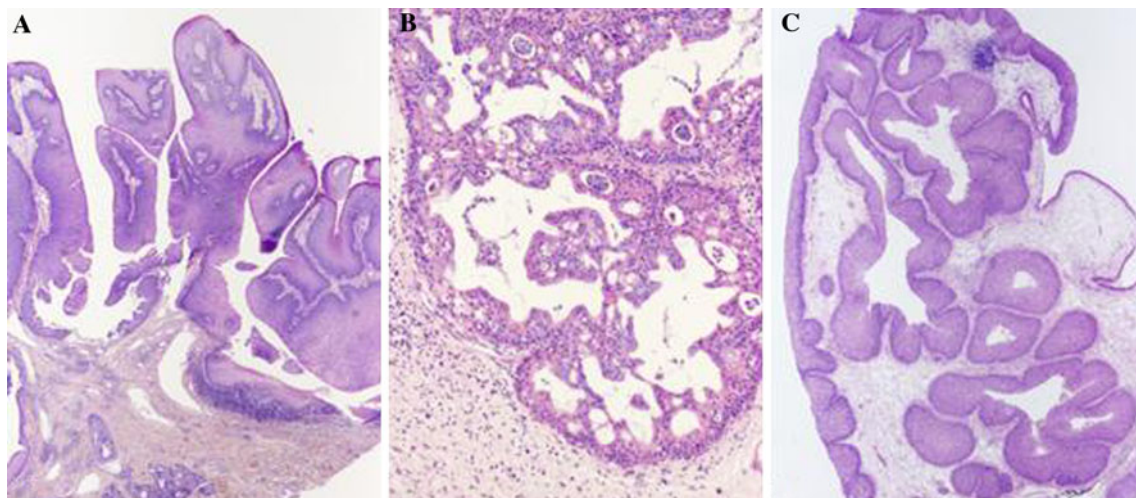


Fig. 1 Schneiderian papilloma histologic subtypes: **a** exophytic papilloma, **b** oncocytic papilloma and **c** inverted papilloma

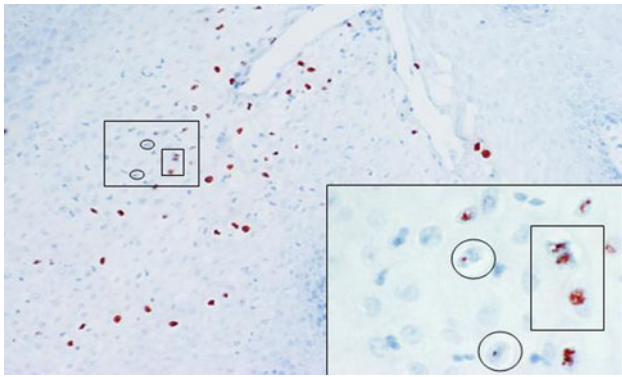


Fig. 2 Chromogenic in situ hybridization (CISH) for HPV. *Inset:* diffuse or episomal signal (*rectangle*) and punctate or integrated signal (*circles*)

ethanol washes and air dried. The slides were then incubated in 10 mM sodium citrate pH 6.0 at 98°C for 40 min followed by a 20 min cool down period. Tissues were then digested with 100 µg/pepsin (Sigma P7012, Sigma–Aldrich, St. Louis, MO) in 0.2 M HCL for 5–12 min (incubation time optimized for each tumor specimen) and were then treated with 0.3% hydrogen peroxide in methanol for 20 min. Next, tissue sections were overlaid with biotinylated Wide Spectrum HPV DNA Probe Cocktail [that detects HPV types 6, 11, 16, 18, 31, 33, 35, 45, 51, and 52 (Dako North America Inc., Carpinteria, CA)] sealed under a cover slip with rubber cement. Slides were placed in a Vysis HYBrite hybridization system (Abbott Molecular, Des Plaines, IL) and heated to 95°C for 5 min to denature specimen and probe DNA and then allowed to hybridize overnight at 37°C. The next day, coverslips were removed under 2 × SSC and then washed 3 × 10 min with 0.1 × SSC at 50°C followed by rinses with 2 × SSC at room temperature. Tissues were then incubated serially with primary streptavidin horseradish peroxidase (HRP) conjugate, biotinyl-tyramide and secondary streptavidin HRP conjugate using a GenPoint™, Catalyzed Signal Amplification System according to supplier instructions (Dako North America Inc., Carpinteria, CA). CISH signals (Fig. 2) were recorded as diffuse (indicative of episomal HPV) or as punctate (indicative of HPV integrated into the cell genome). Using this method, it is possible to detect integrated HPV16 in FFPE preparations of SiHa cells, which are known to contain approximately one copy of the HPV16 genome in chromosome 13q [25]. The detection threshold for episomal HPV is unknown because of difficulties in establishing a model tissue [25, 26].

Immunohistochemistry for HPV L1 Capsid Protein

Immunohistochemistry (IHC) was performed for the HPV major (L1) capsid protein (clone K1H8, Dako North

America, Inc., Carpinteria, CA). Following tissue section dewaxing and high temperature antigen retrieval with sodium citrate (pH 6.0), the antibody (1:50 dilution) was applied to tissue sections for 30 min and after buffer washes, was detected using Envision™ reagents (Dako North America, Inc., Carpinteria, CA) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining.

Immunohistochemistry for p16^{INK4a}

IHC for p16^{INK4a} was performed on all specimens using the CINtec® p16^{INK4a} Histology kit (mtm laboratories, Inc (USA), Westborough, MA) according to supplier instructions and with appropriate positive and negative controls. A positive reaction was defined by strong cytoplasmic and nuclear staining. Four patterns of positive staining were observed and described as follows: pattern a—full-thickness, roman column-like, pattern b—up to the lower ½ of the thickness of the epithelium, pattern c—greater than the lower ½ to full-thickness of the epithelium and pattern d—isolated or sporadic cells distributed throughout the epithelium (Fig. 3).

Statistical Analysis

p values were obtained using Fisher's exact *T* test.

Results

HPV Genotypes Detected by PCR

HPV was detected in 16 of 27 (59.3%) SPs by PCR. HPV positive SPs consisted of 9/19 (47.4%) IPs, 6/6 (100%) EPs [*p* = 0.18], and 1/1 (100%) mixed SP (Fig. 4). HPV was not detected in the single OP in the study. In the PCR positive SPs, both low risk (HPV6 and HPV11) and high-risk (HPV16 and HPV18) genotypes were detected by dot blot hybridization. There were 3 SPs (18.8%) positive for HPV16, 1 with both HPV16 and HPV18 (6.3%), 5 with HPV6 (31.3%) and 7 with HPV11 (43.8%) (Fig. 5). HPV16/18 was detected in 4 of 9 IPs (44.4%) and 0 of 6 EPs (0%) [*p* = 0.10].

HPV Detected by Chromogenic In Situ Hybridization

Of the PCR positive SPs, 7/16 (43.8%) were also positive for HPV by CISH. CISH positive SPs were composed of 5/6 EPs (83.3%) and 1/9 IPs (11.1%) [*p* = 0.01] as well as 1 mixed SP. Punctuate and diffuse signals were seen in 6 of the 7 SPs (1 mixed and 5 EPs) (Fig. 6). Focal diffuse CISH positivity was seen in 1 IP. All CISH positive SPs were either HPV6 or HPV11.

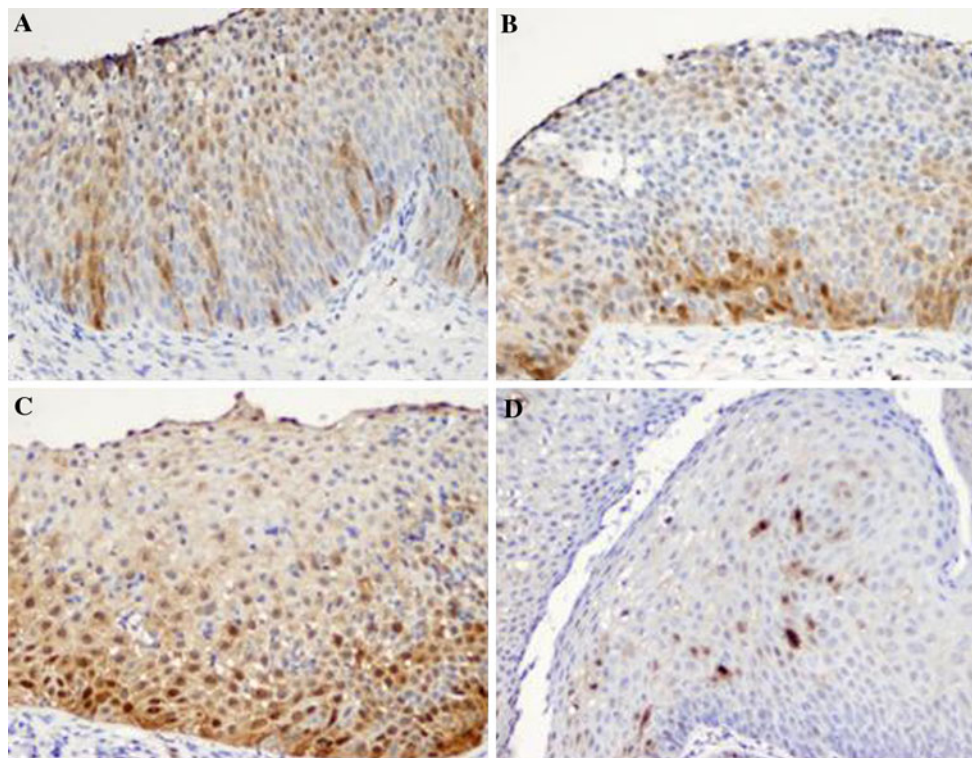


Fig. 3 p16^{INK4a} immunohistochemistry positive staining patterns. **a**—pattern a: full-thickness, roman column-like; **b**—pattern b: up to the lower ½ of the thickness of the epithelium; **c**—pattern c: greater

than the lower ½ to full-thickness of the epithelium; **d**—pattern d: isolated or sporadic cells distributed throughout the epithelium

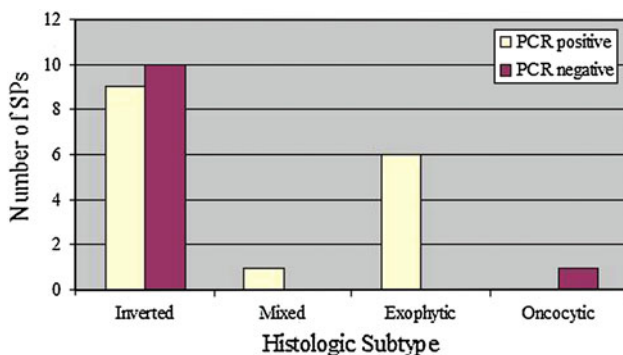


Fig. 4 Correlation of Schneiderian papilloma histologic subtypes with HPV detection by PCR

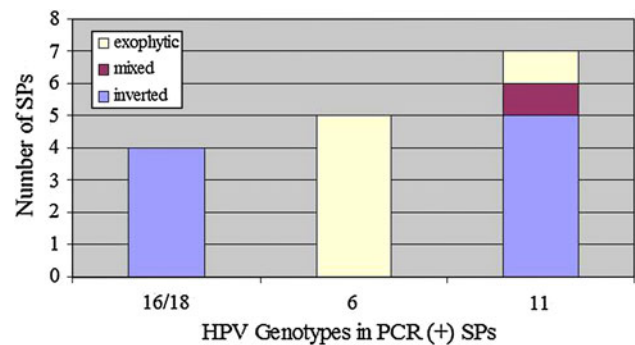


Fig. 5 Correlation of HPV genotypes with schneiderian papilloma histologic subtypes

L1 Capsid and p16INK4a Protein Detection by Immunohistochemistry

IHC for the L1 capsid protein (Fig. 7) was positive in two specimens (1 mixed and 1 EP). Both of these specimens were CISH positive and infected with low risk HPV subtypes (1 HPV6 and 1 HPV11). IHC for p16^{INK4a} (Table 1) was positive in 14/16 (87.5%) PCR positive SPs and in 10/11 (90.9%) PCR negative SPs [*p* = 1.000]. The distribution of the four patterns observed with respect to PCR positivity, CISH positivity, HPV genotypes and histologic subtype is

shown in Table 1. A combination of staining patterns was the most common staining pattern seen in 12 of 28 SPs (42.9%). A combination staining pattern is defined as SPs not having a single A, B, C or D pattern but rather demonstrating more than one of the previously defined staining patterns for example an SP may show patterns A, B and C. No SP demonstrated only pattern B or pattern C.

Schneiderian Papillomas with Dysplasia

Of the 2 EPs displaying mild dysplasia, both were positive for HPV by PCR and ISH. HPV was not detected in the IP

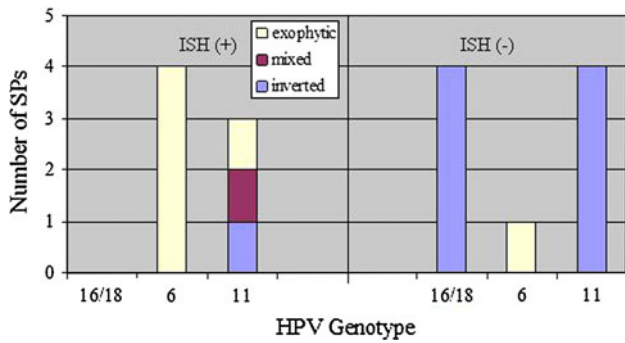


Fig. 6 Distribution of HPV genotypes and Schneiderian papilloma histologic subtypes in CISH positive (+) and CISH negative (-) Schneiderian papillomas

with multifocal moderate dysplasia, however, HPV was detected by PCR only in the IP with focal severe dysplasia. Low risk HPV was detected in the 3 of 4 SPs (75%) displaying dysplasia (2 HPV6 and 1 HPV11).

Discussion

In this study, the prevalence of HPV in SPs was 59.3%. HPV was detected in higher frequency in EPs (100%) as compared to IPs (47.4%), which is consistent with current literature [6–10]. The presence of HPV DNA in 100% of EPs by PCR suggests that HPV may be the major causative factor in these SPs. The lower detection rate of HPV in IPs suggests that the pathogenesis may be multifactorial; alternatively there may be an HPV pathway for a subset of IPs versus a non-HPV pathway as has been found in vulvar squamous cell carcinoma [27].

In an attempt to explain the disparate detection rates between IPs and EPs, Lawson et al. suggested a “hit and

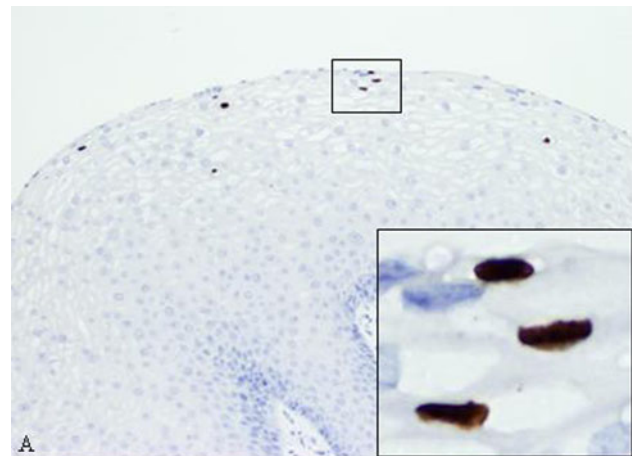


Fig. 7 Immunohistochemistry for the HPV L1 capsid protein. Positive staining defined by strong nuclear positivity

run” mechanism [10]. The latter was first described by Skinner (1976) which proposes that a virus may initiate cell transformation, and then disappear without leaving viral “footprints” [28]. This hypothesis was extended to IPs suggesting that the non-keratinizing nature of IPs lends itself to reduced viral replication and with epithelial shedding the virus is lost. In contrast, the hyperkeratotic epithelium of EPs is permissive for replication accounting for the higher detection rates of HPV in EPs [10]. A contradiction to Lawson’s hypothesis is that usually only a small number of EPs exhibit surface keratination [3, 29]. Nevertheless, the “hit and run” mechanism remains the most plausible explanation for the lower detection rate of HPV in EPs.

HPV was not detected in the single OP, similar to other studies [6–9]. The majority of SPs (75%) were infected with low-risk HPV6/11 genotypes. Interestingly, 3 of 4 SPs

Table 1 p16^{INK4a} immunoreactivity in Schneiderian papillomas according to HPV status

	A	B	C	D	A&B	A&C	A&D	B&C	A&B&D	Negative	Total
PCR (+)	5	0	0	1	2	5	1			2	16
PCR (+)/ISH (+)	1	0	0	1		4				1	7
PCR (+)/ISH (-)	4	0	0		2	1	1			1	9
PCR (-)	3	0	0	2				2	2	2	11
HPV16/18	2	0	0		2						4
HPV11	2	0	0	1		2				2	7
HPV6	1	0	0			3	1				5
EPs	1	0	0	2		3	1				7
IPs	6	0	0	2	2	2		2	2	3	19
Mixed		0	0							1	1
OP	1	0	0								1

p16^{INK4a} staining patterns: A—Full-thickness, roman column-like; B—Up to the lower ½ of the thickness of the epithelium; C—Greater than the lower ½ to full-thickness of the epithelium; D—Isolated or sporadic cells distributed throughout the epithelium

(2 EPs and 2 IPs) with dysplasia were positive for low-risk HPV genotypes. However, the four SPs with high-risk HPV 16/18 genotypes were IPs without dysplasia. There was no statistical difference between high-risk HPV genotype detection in IPs (44.4%) as compared to EPs (0%) [$p = 0.10$]. Given the association of IPs with malignancy, it seems logical to assume that high-risk HPV genotypes would be found in greater frequency in IPs as compared to EPs.

The integration of viral specific oncogenes *E6* and *E7* is characteristic of high-risk HPV infections in high-grade pre-invasive and invasive cervical neoplasia and may be critical for the conversion to a malignant phenotype [18]. *E7* degrades RB and releases p16^{INK4a} from negative feedback control, resulting in p16^{INK4a} upregulation. This relationship between p16^{INK4a} overexpression and HPV infection has made p16^{INK4a} a useful surrogate marker for high-risk HPV infection in tonsillar carcinoma and cervical neoplasia [19, 20]. Although most SPs are benign with low-risk HPV genotypes most commonly detected [5], the relationship with p16^{INK4a} has not been fully explored. Two previous studies investigated p16^{INK4a} and SPs [21, 22]: the first examined p16^{INK4a} and p53 in the dysplasia-carcinoma pathway in SPs and the second investigated whether p16 could be used to differentiate EPs from squamous papillomas [21, 22]. Our interpretation of positive p16^{INK4a} staining was based on strong nuclear and cytoplasmic staining and demonstrated that there was no statistical difference between p16^{INK4a} staining in PCR positive (87.5%) versus PCR negative (90.9%) SPs [$p = 1.000$]. These findings suggest that p16^{INK4a} cannot be used as a surrogate marker for HPV in SPs.

HPV is increasingly recognized as important in the etiology of many head and neck tumors and has patient management implications [30]. Thus, there is a need for a standardized HPV assay or assays for the definitive detection of clinically significant HPV. A distinction has been made between ‘driver’ HPV that is causal for a tumor, and ‘passenger’ HPV that is an incidental bystander infection unrelated to tumorigenesis [31]. PCR assays allow the sensitive identification of HPV in a tumor; however, the very sensitivity of PCR may confound the discrimination of driver from passenger HPV. The visualization of HPV directly in cells and tissues by CISH assays is consistent with the detection of driver HPV; however, the possibility of CISH sub-threshold driver HPV cannot be entirely excluded as CISH data may depend on tissue fixation, assay protease treatments, HPV probe character, CISH detection strategy, and assay application in a given laboratory. p16^{INK4a} IHC is the simplest test to apply and has been reported not only as an efficient HPV surrogate marker for tonsillar carcinomas but as a test with good clinical outcome correlates [30]; HPV testing by CISH was

also reported as a suitable approach [30]. Our study does not support the application of p16^{INK4a} IHC to papillomas; a combination of HPV screening and typing by PCR and CISH may be the best approach for HPV testing of SPs at the present time.

In summary, this study demonstrates a strong association between HPV and EPs, whilst its role in IPs is less well-defined. Alternatively, HPV could be a single factor contributing to the multifactorial pathogenesis of IPs. Finally, p16^{INK4a} is not a useful surrogate marker for HPV detection in SPs.

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